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INSTITUT PHYTOPATHOLOGIQUE BENAKI

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POST-INFECTION DEVELOPMENT OF *MELOIDOGYNE INCOGNITA* CHITWOOD, 1949 (NEMATODA: HETERODERIDAE)[1]

A. C. TRIANTAPHYLLOU AND HEDWIG HIRSCHMANN [2]

The first complete account of the life cycle of a *Meloidogyne* species was published in 1941 by Christie and Cobb (3). These investigators distinguished 5 developmental stages separated by 4 molts. The first molt takes place within the egg. The second stage larvae hatch and enter the roots of the host plant. After a relatively long feeding period, during which the vermiform infective larvae attain a saccate shape, the second and third molts occur in rapid succession. The fourth molt follows and gives rise to adult males and females.

Prior to the investigation of Christie and Cobb, the only comprehensive study on the life cycle of the root-knot nematode was that by Nagakura (7). He believed that the second larval molt occurs shortly after the larvae have entered the roots, and that the change in their shape from vermiform to saccate takes place after the second molt. The species of *Meloidogyne* which Nagakura and Christie and Cobb studied are not known since their work was done before Chitwood's revision of the genus (2).

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Contribution from Plant Pathology, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 1104 of the Journal Series.

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This study constitutes a part of a thesis submitted by the senior author to the Graduate School of North Carolina State College in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

The life cycle of a root-knot nematode closely resembling *M. incognita acrita* Chitwood, 1949 was studied by Ritter and Ritter in 1958 (9) who agreed with Nagakura in that the second molt takes place 3 to 4 days after the infective second stage larvae have entered the roots of the host plant. The third and fourth molts occur in close succession.

The development of *M. javanica* (Treub, 1885) Chitwood, 1949 and *M. hapla* Chitwood, 1949 was recently investigated by Bird (1), who agreed with Christie and Cobb regarding the stages of development and reported the approximate time necessary for the comletion of each stage.

In regard to sex differentiation, Nagakura stated that the first and second stage larvae (second or second and third stages as defined by Christie and Cobb) are sexually undifferentiated. The male larva is distinguishable by its characteristic vermiform body shape only in the third stage (fourth stage according to Christie and Cobb). Christie and Cobb, on the other hand, believed that the sexes can be distinguished at the end of the second larval stage.

Sexual differentiation becomes apparent during the second larval stage in the citrus nematode, *Tylenchulus semipenetrans* Cobb, 1913 (5); early in the third larval stage in the beet-cyst nematode, *Heterodera schachtii* Schmidt, 1871 (8); and early in the fourth larval stage in the sheath nematode *Hemicycliophora arenaria* Raski, 1958 (6). In most other plant parasitic nematode species the exact larval stage in which the sex can be distinguished morphologically is not known.

In the studies reported herein an attempt was made to distinguish morphologically the developmental stages and the sex of larvae

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LEGEND FOR FIGURES

ex. p.	= excretory pore	per. patt.	= perineal pattern
gen. pr.	= genital primordium	rect.	= rectum
gon.	= gonad	rect. gl.	= rectal gland
hyp.	= hypodermis	spic.	= spicule
int.	= intestine	test.	= testis
med. blb.	= median bulb	ut.	= uterus
mlt.	= molt	vag.	= vagina
n.	= nucleus	vas def.	= vas deferens
oes. gl.	= oesophageal gland	vlv.	= valve
ovr.	= ovary		

of *M. incognita* and to determine approximately the duration of each larval stage under certain environmental conditions. A preliminary report has been published (11).

MATERIALS AND METHODS

Tomato seedlings of the susceptible variety Rutgers were propagated in 2″ pots in steam-sterilized soil. When the seedlings had reached a height of about 10 cm, a water suspension of approximately 200 larvae of a single egg mass isolate of *M. incognita*, was injected with a fine pipette into the soil close to the roots. Forty-eight hours later the roots were washed to remove any superficial larvae, and the seedlings were transplanted into steam sterilized soil in 6″ pots. The pots were placed in a greenhouse maintained at approximately 29° C.

At 2-day intervals some plants were washed free of soil. The galled portions of their root systems were stained with boiling acid fuchsin lactophenol (4) and transferred to pure lactophenol. All nematodes were later dissected out of the roots and mounted on slides in pure lactophenol for microscopic examination.

In addition, young tomato seedlings were transplanted into 6″ pots containing soil lightly infested with larvae of the same isolate. These plants were grown for 2 months to permit development of a second generation of the parasite in roots of the same plants. The roots were then washed free of soil and treated in the same way as the roots of the young seedlings. This material was used to study the developmental morphology of males which were absent in roots of young plants.

EXPERIMENTAL RESULTS

1. Development in Relation to Nematode Morphology and Anatomy.

In general, the development of *M. incognita* was found to be similar to that described by Christie and Cobb (3). After entering the roots of the host plant, the vermiform infective second stage larvae (Fig. 1A) start feeding, and in a few days grow thicker without increasing in length (Fig. 1B). The genital primordium at this stage lies at nearly 65-70% of the body length and consists of a small group of cells. The sex is still not recognizable. The larvae at this stage can be referred to as developed, sexually undifferentiated second stage

larvae. They are distinguished from the infective larvae by the change in body shape and slight growth of the genital primordium. During the following several days the larvae continue to increase in thickness and soon the genital primordia attain a shape characteristic of their sex.

In female larvae cell division and enlargement is initiated at 2 sides of the genital primordium and the gonad appears V-shaped (Fig. 1C). The 2 branches, generally directed anteriad, increase in length while the gonad moves towards the posterior end of the body. Finally the gonad is located in front of the rectum close to the body wall (Fig. 2A). At this time the second molt takes place.

In male larvae cell division and enlargement is initiated at only one side of the genital primordium and the gonad attains a straight cylindrical shape (Fig. 1D)[1]. It moves slowly posteriad and its basal part becomes slightly swollen (Fig. 3A). Its orientation at this time is found to be rather variable, i.e. the cylindrical part is directed mostly anteriad, but sometimes posteriad or laterad. It has been observed that the gonad moves posteriad much slower in the male than in the female larvae. Shortly before the second molt of the male larvae the basal part of the gonad is situated close to the rectum without joining it (Fig. 3A).

The female second stage larvae possess 6 well developed rectal glands[2] (Fig. 1C). These glands have not been observed in male larvae.

The excretory pore opens in both sexes far back from the median oesophageal bulb. The hypodermis is highly developed.

The general size, particularly the width of the larvae, is very variable depending perhaps on the amount of nutrition available. No constant correlation can be established between the width of the larvae and their sex. Male larvae show a greater variation in body size than female larvae. Under conditions unfavorable for the development

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[1] The case of male individuals with 2 gonads has been discussed in relation to sex determination (10). Males with 2 testes are considered to develop from female second stage larvae through sex reversal.

[2] After this paper was submitted for publication, a detailed investigation of the rectal glands in four *Meloidogyne* species was published (Maggenti and Allen, Proc. Helminthol. Soc. Wash. D. C. 27 : 4-10). The present independent study supports Maggenti and Allen's observations on the occurrence of rectal glands in developing female larvae and adult females. In our studies, rectal glands were not observed in male larvae and these glands gradually degenerated and finally disappeared in female larvae which developed into males following sex reversal.

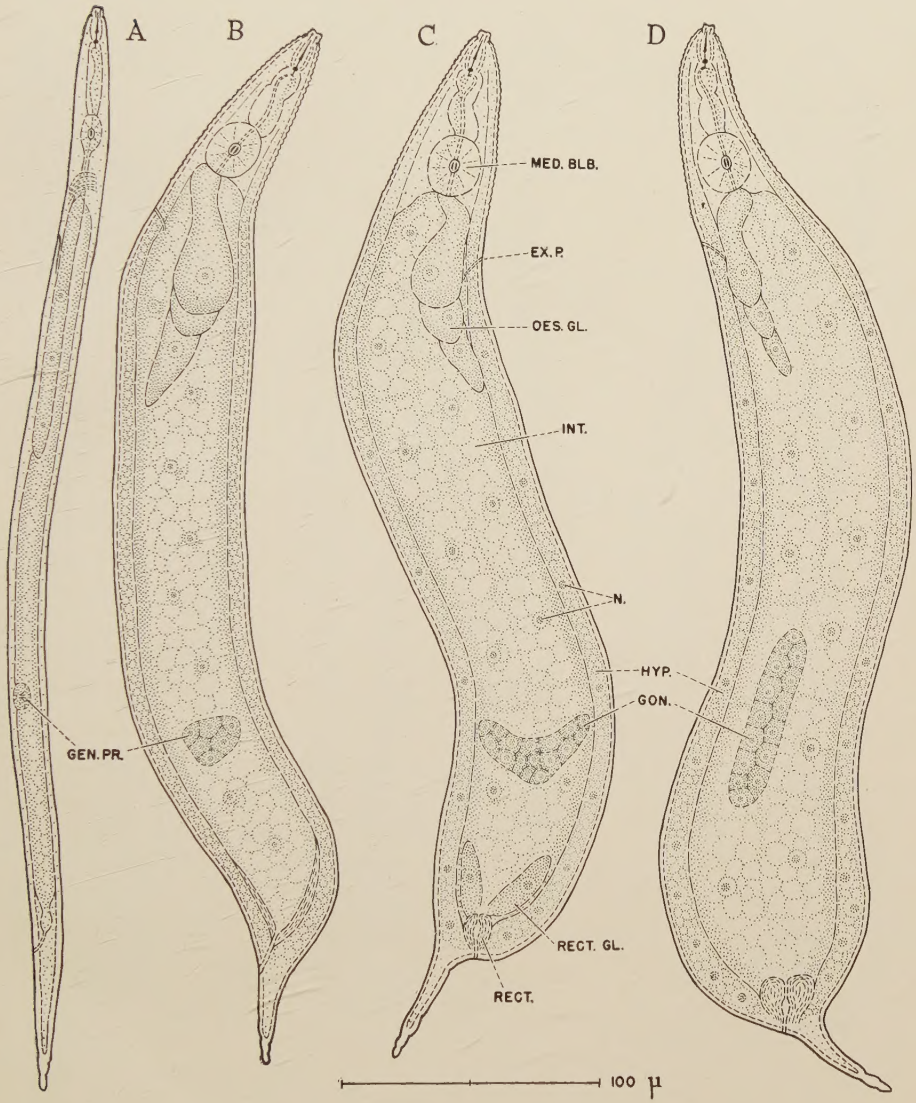

Figure 1. Development of *M. incognita*. A — second stage infective larva; B — developed, sexually undiffentiated second stage larva; C — early second stage female larva; D — early second stage male larva.

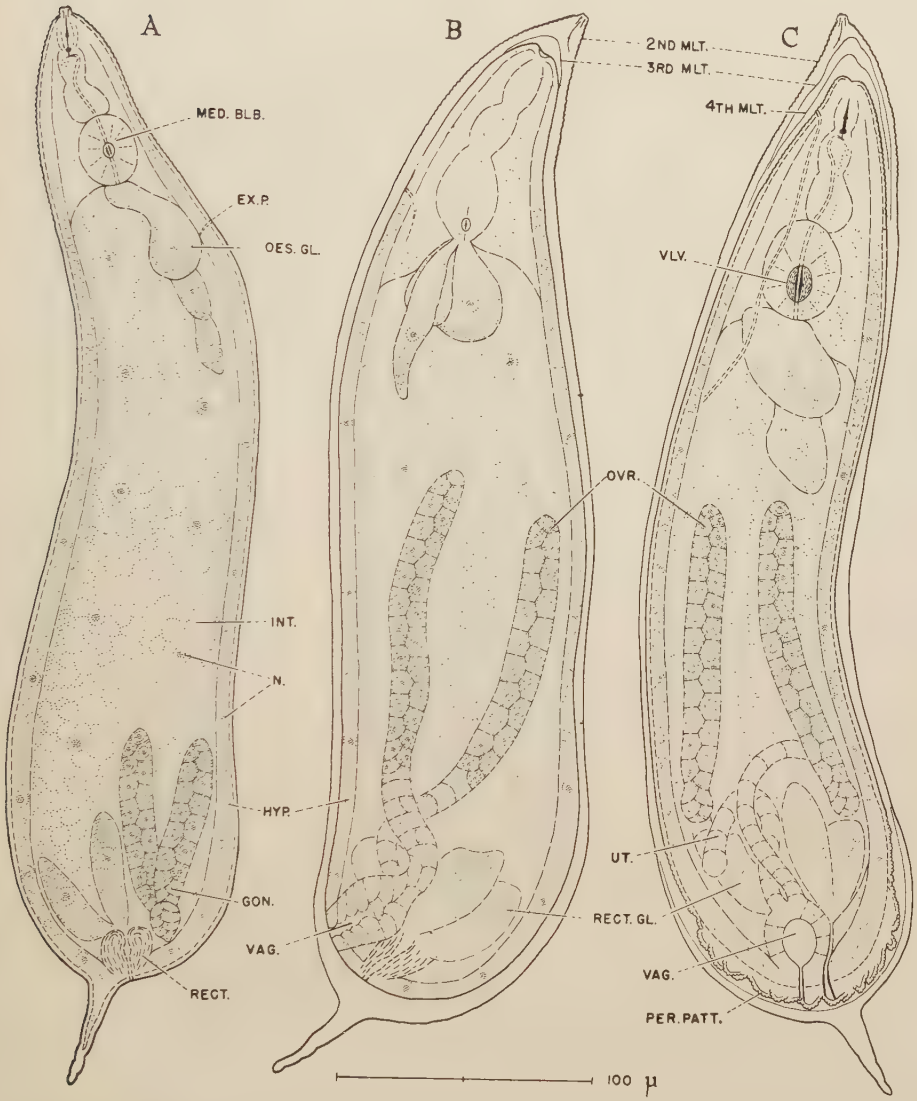

Figure 2. Development of *M. incognita.* A — second stage female larva shortly before the second molt; B — fourth stage female larva; C — adult female shortly after the fourth molt.

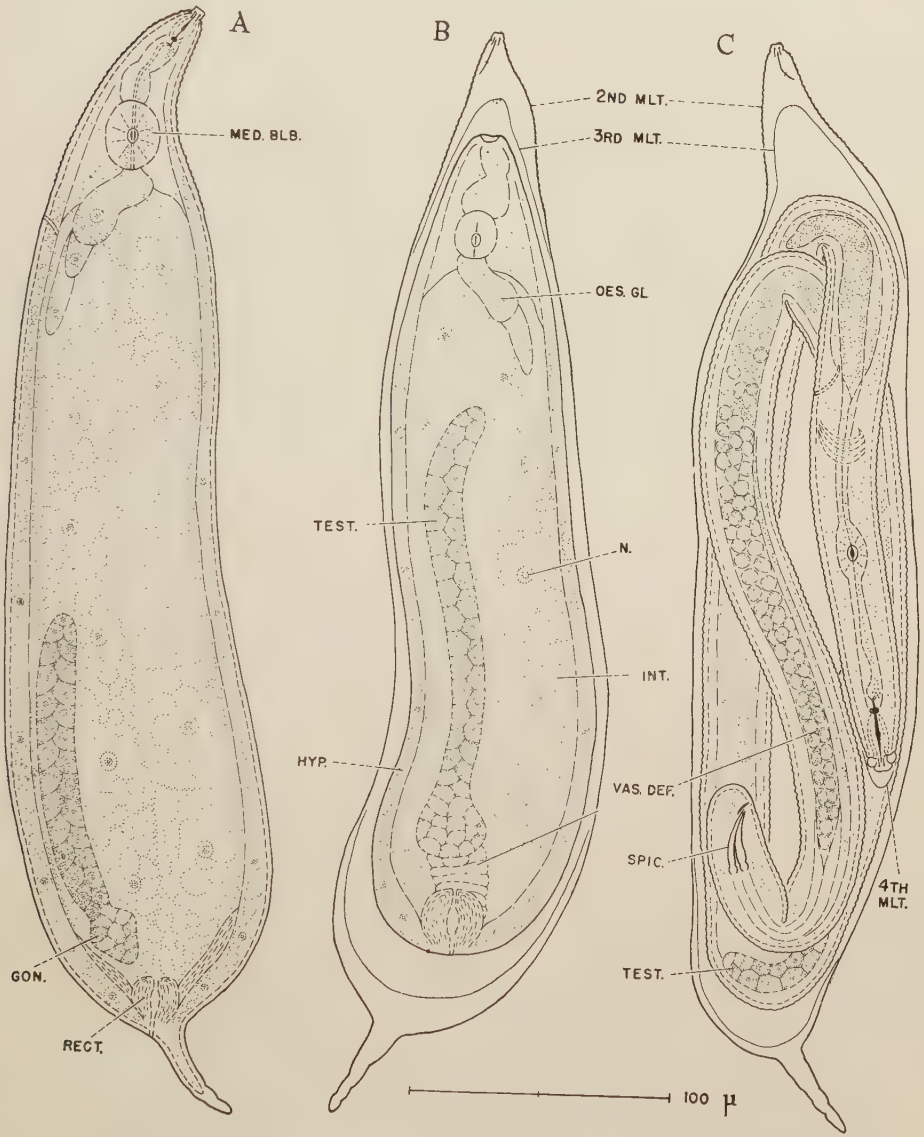

Figure 3. Development of *M. incognita.* A — male second stage larva shortly before the second molt; B — early fourth stage male larva; C — adult male shortly after the fourth molt.

of the nematode, advanced second stage male larvae may be thinner or much thicker than female larvae.

The second molt gives rise to the third stage larva which can be easily distinguished from the late second stage larva in that it is enclosed in the second larval cuticle, it possesses no stylet which disappears during the second molt, and the posterior end of the body is rounded, without tail spike.

It may be noted here that the body contents of second stage larvae usually contract and separate from the cuticle when treated with boiling acid fuchsin lactophenol, thus giving the impression of a molt. Such individuals may be mistaken for third stage larvae. Careful examination, however, reveals that the cuticle is separated all around the body, but not in the head region. Also a distinct stylet is present, and the body contents in the posterior end extend into a tail spike.

The third larval stage lasts probably only a few hours in both sexes. It is succeeded by a third molt during which a very thin cuticle, mostly visible only in the head region of the nematode, is shed.

The fourth larval stage extends over a relatively longer period. Early in this stage the valve of the median oesophageal bulb, that had disappeared shortly after the second molt, is reformed in the posterior part of the bulb (Figs. 2B and 3B). Stylet and oesophageal duct are absent. The excretory pore opens nearly at the level of the posterior part of the median oesophageal bulb. During this stage the gonads grow considerably in both sexes. The uterus and vagina in the female and the vas deferens in the male begin to differentiate. The rectal glands in female larvae are enlarged. The male larvae undergo metamorphosis and at the end of this stage attain a cylindrical, elongate shape.

The fourth molt follows and the adults appear enclosed in the larval cuticles of the previous 3 stages (Figs. 2C and 3C). The stylet which was absent in the third and fourth stages reappears in the adults of both sexes.

In the adult female the oesophageal duct becomes distinct and the valve of the median oesophageal bulb attains a considerably larger size than in the previous stages. The excretory pore opens at the level of the stylet shaft. The rectal glands are well developed. The characteristic striations of the perineal pattern appear as soon as the cuticle of the young female becomes distinct. In young females stained with acid fuchsin lactophenol the body contents contract and

separate from the cuticle, thus the perineal region appears as shown in Fig. 2 C. Soon the females begin to feed and increase in size. The cuticles of the previous molts disappear shortly afterwards. Egg-laying starts later after the gonads have completed their development.

In the male the gonad is well developed and sperm is produced shortly after the fourth molt when the individual is still enclosed in the larval cuticles (Fig. 3C).

2. Development of Females in Relation to Time.

Data relative to the time required for the development of *M. incognita* in tomato roots at an approximate temperature of 29° C are summarized in Table I.

TABLE I

Development of *Meloidogyne incognita* on tomato (var. Rutgers) at approximately 29° C.

Sample No	Age (days after penetration)	Number of individuals examined	Larval stage (larvae %)					Females (%)[c]	
			2nd			3rd	4th	without eggs	with eggs
			infective	A[a]	B[b]				
1	7— 9	73	82.2	16.4	1.4	—	—	—	—
2	9—11	150	16.7	73.3	10.0	—	—	—	—
3	11—13	144	4.8	9.7	80.6	2.1	2 8	—	—
4	13—15	74	6 8	9.5	35.1	8.1	32.4	8.1	—
5	17—19	92	—	5.5	13.0	5.4	25.0	51.1	—
6	19—21	145	—	4.1	13 8	1.4	5.5	72 4	2.8

[a] A = developed but still sexually undifferentiated 2nd stage larvae.
[b] B = sexually differentiated 2nd stage larvae.
[c] = Under the conditions of this experiment no males developed.

It is obvious that larvae which have been in tomato roots for the same period of time are not necessarily in the same stage of development. In sample N° 6, e.g., a range of different stages from sexually undifferentiated second stage larvae to adult egg-laying females was found.

During the first 13 days after penetration (samples 1 to 3) most of the larvae were still in the second stage. Within the period from

the 11th to 13th day, however, a few larvae had undergone the second or the second and third molts. The first adult females appeared 13-15 days after penetration (sample 4). Therefore, the second, third, and fourth molts in female larvae occurred within a period of 4 days, most probably 2 to 3 days. The first mature egg-laying females were found 19 to 21 days after penetration (sample 6).

Although some second stage larvae were sexually differentiated within 7 to 9 days (sample 1), the majority of them showed sexual differentiation 11 to 13 days after penetration (sample 3). Some larvae remained sexually undifferentiated even after a period of 19 to 21 days (sample 6).

A correlation was observed between the extent of larval development and the relative number of larvae present in a certain portion of a root. When numerous larvae were crowded in a small root portion, their development was significantly retarded as compared to larvae of the same age that had entered a particular root part singly.

Development of males in relation to time was not studied. This subject is rather complex since it is related to the mechanism of sex determination which in turn is affected by the environment (10).

DISCUSSION

Findings in regard to post-infection development of *M. incognita* larvae differ from those presented by Ritter and Ritter (9). It is possible that species or populations of *Meloidogyne* from different geographical areas may differ in their developmental pattern.

According to Christie and Cobb (3) the sex of the root-knot nematode can be distinguished in the advanced second stage larvae on the basis of body shape. This character, however, is of doubtful value in differentiating the sexes in *M. incognita*. The great variation in shape and relative growth of the advanced second stage larvae of this species makes any inference on these characters as to their sex difficult. The several characteristics used in these studies to distinguish the sex of advanced second stage larvae are easily visible in acid fuchsin lactophenol stained material. Male larvae possess one gonad and apparently do not have rectal glands, whereas female larvae possess 2 gonads and well developed rectal glands. Under certain conditions favorable for sex reversal (10), distinction of the sex of the larvae on the basis of the above mentioned criteria is rather difficult since in addition to the above 2 types all intermediate forms can also be found.

A striking feature in the development of *M. incognita* seems to be the great variation in degree of development among individual larvae. This variation may result from differences in rate of development during the second larval stage when the nematodes are feeding. In the female it may also result from differences in rate of development from the period of the fourth molt to the time of maturity. Tyler (12) found similar variation in development of root-knot nematode larvae and stated that this variation may be related to their nutrition. It is possible that nutritional conditions are not similar for all larvae present in the root system of a plant, and this may account for differences in their rate of growth and development. Crowding and position of each individual larva in the root tissues may be two factors affecting the amount of food available to the nematode.

SUMMARY

In studies of post-infection development of *Meloidogyne incognita* the morphology in the various larval stages was described and the time required for the development of the female larvae under certain environmental conditions was determined.

The various larval stages were well defined with respect to several morphological characters. Sex was distinguishable in half-grown second stage larvae. Female larvae possessed 2 gonads and 6 rectal glands, whereas male larvae had one gonad and apparently no developed rectal glands. In addition, the male gonad during its development moved posteriad much slower than the female gonad.

In female larvae the second molt was first observed 11 to 13 days after penetration of the larvae into the roots of susceptible tomato (var. Rutgers) held at approximately 29° C. The third molt followed within a few hours and the fourth molt occurred 2-4 days later. Adult females appeared 15 days after penetration of the roots and 6 days later some eggs were laid. Considerable variation was observed in the degree of development among larvae present in the roots for the same period of time. Larval development was significantly retarded where numerous larvae were crowded in a small root portion.

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SEX DETERMINATION IN *MELOIDOGYNE INCOGNITA* CHITWOOD, 1949 AND INTERSEXUALITY IN *M. JAVANICA* (TREUB, 1885) CHITWOOD, 1949 [1]

A. C. TRIANTAPHYLLOU [2]

The mechanism of sex determination in the genus *Meloidogyne* Goeldi, 1887 has not been investigated in detail. Knowledge on this subject is limited and is based mainly on observations rather than on results of experimental investigations. An exception is Tyler's work (18) on sexuality of the root-knot nematode. With regard to the occurrence of males, Tyler found that in roots of tomato plants growing in an agar medium, males appeared under unfavorable conditions. She suggested that conditions of nutrition may be responsible for changes in the physiological balance of certain individuals during an early stage of their development. In addition Tyler reported observations which suggest that not only in the root cultures but also in the field, males occur under adverse conditions. In support to her conclusions she mentioned similar findings by Molz and Hornburg regarding the occurrence of males in the sugar-beet nematode, *Heterodera schachtii* Schmidt, 1871. Linford (12) agreed with Tyler that unfavorable conditions cause an increase of the percentage of males. He found relatively abundant males in cowpea leaves, especially in small veins and near the margins where weak development of females indicated poor nutrition.

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[2] Nematologist, Benaki Phytopathological Institute, Kiphissia, Athens, Greece. This study constitutes a part of a thesis submitted to the Graduate School of North Carolina State College in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

There are numerous statements in the literature that males of root-knot nematodes were found to be abundant in some cases and rare or absent in others. Excessive numbers of males were produced by 2 populations of *M. incognita acrita* in cotton roots in which no females developed to maturity according to van der Linde (11). The same investigator noticed that males and immature forms were more numerous than adult females in the roots of a commercial variety of *Ricinus communis* L. Dropkin (6) estimated the percentage of males found in large and small galls of several soybean varieties infected by *M. incognita incognita*, *M. incognita acrita* and *M. arenaria* (Neal, 1889) Chitwood, 1949. He found no correlation between male abundance and gall size. Furthermore, he related abundance of males to suitability of the host. Favorable host varieties had few males, whereas unfavorable host varieties had percentages of males varying from low to high. He suggested that male development may follow a complex pattern and that it may be influenced by specific conditions present in some hosts. According to Ellenby (7) many more males than females developed in roots of potato plants heavily infected by *Heterodera rostochiensis* Woll., 1923, whereas in lightly infected roots the male to female ratio was low. Golden and Shafer (8) found many males of *H. schachtii* in a resistant line of *Chenopodium album* L. in which no females developed to maturity. Smaller numbers of males were present in a susceptible line but even smaller numbers were found in 3 other resistant lines of the same plant species.

In *Mermis subnigrescens* Cobb, 1925 which is an insect parasite, Christie (5) has shown that sex is mainly determined by environment. When 1 to 3 nematodes were present in one grasshopper they all became females. When 4 to 14 were present they became either females or males and when 15 or more, nearly all developed into males. In this species females can reproduce parthenogenetically and the function of the male is not known.

Intersexes have been reported in relatively few genera of nematodes. Literature on this subject up to 1923 has been reviewed by Steiner (15) who also made a thorough study on intersexes of *Agamermis decaudata* Cobb, Steiner and Christie, 1923. He found that in this species intersexes were as numerous or even more numerous than normal females. Literature up to 1955 has been reviewed by Hirschmann and Sasser (10) who also reported the occurrence of intersexes in the genus *Ditylenchus* Filipjev, 1934. Chitwood in 1949 (4) reported male intersexes in *M. javanica*. This is the only report of male inter-

sexes in nematodes. All other intersexes of nematodes reported are females showing in addition male characters.

The mechanism of intersexuality in other nematode species has been explained in a number of ways. Steiner (15) suggested a mechanism similar with that of *Lymantria dispar* L. in which, as Goldschmidt (9) has found, intersexuality results from hybridization of closely related genotypes.

Christie (5), after considering his findings on the effect of the environment on sex determination in *Mermis subnigrescens,* stated that the occurrence of intersexes probably is in some way linked with sex determination. He postulated that when one female is developing in the same insect with many males, it is continuously under the influence of some environmental force tending to stimulate maleness. This force may be sufficient to induce development of secondary male characters and thus the female becomes an intersex.

In these studies an attempt was made to investigate the mechanism of sex determination and the effect of several factors on sexual differentiation of larvae of *M. incognita. M. javanica* was included in order to relate intersexuality occurring in this nematode species to the phenomenon of sex reversal proposed as part of the mechanism of sex determination of *M. incognita.* A preliminary report has been published (16).

MATERIALS AND METHODS

Tomato seedlings (var. Rutgers), 10 to 15 cm high, were individually inoculated with approximately 200 larvae of a single egg mass isolate of *Meloidogyne incognita.* Each plant was placed in a small glass beaker and a water suspension containing the larvae was poured over the roots. The roots were then covered with a 50-50 mixture of steam-sterilized soil and sand. Five days later the seedlings were washed to remove any lavae adhering to the surfaces of the roots or present in the surrounding soil. They were transplanted into 6" pots filled with steam-sterilized soil and placed in a greenhouse at approximately 29° C. Samples of 5 to 10 plants were taken at intervals of 5 to 10 days. The plants were washed free from soil and the galled root portions removed and divided into 2 representative samples (halves). A special effort was made to select equally galled tissues for each half to minimize variation due to differences in severity of infection.

One half of the galled root tissue was stained for 4 minutes in boiling acid fuchsin lactophenol and was then transferred into pure lactophenol for clearing. The other half was placed in a small beaker in a moist chamber and kept at 19° C for 40 days.

All nematodes present in the stained roots were dissected out, mounted on slides in pure lactophenol and examined under the compound microscope to determine their developmental stages and sex. The second stage larvae were classified into 3 categories: (a) infective larvae; (b) developed 2nd stage, sexually undifferentiated lavae; and (c) 2nd stage sexually differentiated lavae (17).

A small quantity of water was added every second day to each beaker containing the incubated roots. After a short period of agitation, the water was poured off in a syracuse watch class and examined for the presence of males.

In the first experiment males obtained from incubated roots were counted, and the body length of some of them was measured. In subsequent experiments, the males were stained for 1 minute in hot acid fuchsin lactophenol, mounted on slides in pure lactophenol, and their body length and number of testes were recorded.

EXPERIMENTAL RESULTS

Data obtained from the examination of stained and incubated roots are summarized in Table I.

In sample 5 all nematodes found in the stained roots were adults. In sample 6 new infection by larvae of the second generation was observed. In order to study the development of larvae of the second generation separately from larvae of the first generation, samples 6 to 11 were treated in a different way than samples 1 to 5. In samples 1 to 5 one half of the galled roots was incubated and the other was stained as previously described. Beginning with No 6, however, each sample consisted of the root system of a single tomato plant. A few roots containing new galls, evidently caused by larvae of the second generation, were stained, whereas the rest of the root system was incubated. Therefore the number of males obtained from the incubated roots of samples 6 to 11 cannot be compared with the number of males observed in the stained roots, since the two parts were originally unequal.

In the first generation very few males or male larvae (larvae with one gonad) were found in the stained roots (Table I, samples 1 to 5).

TABLE I

Development of *Meloidogine incognita* on tomato at 29° C and numbers of males obtained from infected roots incubated for 40 days in moist chambers at 19° C.

Sample No	Development Days [a]	Incubated roots	Stained roots											
		Males No	2nd stage larvae undifferentiated		No females					No males				Males %
					Larval stages			Adults		Larval stages			Adults	
			Infective	Developed	2nd	3rd	4th	Without eggs	With eggs	2nd	3rd	4th		
1	8—13	23	2	22	64	6	26	—	—	—	—	—	—	0
2	13—18	9	2	14	8	—	1	124	—	1	—	—	—	0.7
3	18—23	2	—	—	5	—	—	79	16	1	—	1	—	2.0
4	23—28	1	—	—	—	—	—	11	130	—	—	1	—	0.7
5	27—32	1	—	—	—	—	—	—	156	—	—	—	1	0.6
6	32—37	—	84	—	—	—	—	—	—	—	—	—	—	—
7	37—42	57	501	63	2	—	—	—	—	—	—	—	—	0
8	42—47	361	115	51	30	2	9	5	—	4	—	—	—	8 0
9	48—53	791	65	47	36	5	24	77	—	28	—	1	—	17.0
10	55—60	1258	43	17	14	1	2	61	—	23	4	14	5	37.0
11	66—71	1363	49	21	15	2	8	72	21	96	5	18	29	55.6

[a] period from penetration of larvae of the first generation into the roots to incubation or staining.

In the second generation males became more abundant. In sample 8, e.g., males constituted 8% of the sexually differentiated individuals. This percentage increased reaching a maximum of 55.6% in sample 11 when the experiment was completed.

Influence of crowding of the larvae on male development. It was postulated that the increase in the percentage of males from the first to the second generation could be attributed to one or both of 2 main factors: (1) changes in the physiology and chemical composition of the plants associated with ageing, or (2) crowding of individuals resulting from heavier infection of the roots by the numerous larvae of the second generation.

The latter possibility was studied during the examination of the stained material of sample 11. For this purpose a camera lucida drawing of each individual rootlet was made. The nematodes present in each rootlet were dissected out and mounted on a slide. In this manner it was possible to compare sex ratio with intensity of infection as well as with position of nematodes in the roots. Shape and relative size of several rootlets are illustrated in Fig. 1. The number of sexually undifferentiated and differentiated individuals in each part of the rootlet and the percentage of males are also given. It is apparent that the apical portion of the rootlets contained a high percentage of males. In these parts the nematodes were very crowded. Smaller numbers of nematodes and smaller male percentages or no males at all were present in parts of the same rootlets away from the apical portion. Similar observations have been made in many other root examinations during these studies.

The high percentage of males in the apical portion may be explained on the basis of intensity of infection and host response to the nematode attack. When numerous lavae enter the apical portion of a rootlet, changes in root metabolism and food translocation may create conditions unfavorable for both root growth and nematode development. The apical meristem ceases to grow and most of the nematode larvae differentiate as males. In lighter infections physiological disturbances in apical portions of rootlets are less pronounced. The apical meristem continues to grow and conditions are normal for development of the nematodes. Most of the nematode larvae in such cases differentiate as females. According to this explanation most of the males would be expected to be found in the apical galls of the roots of a host plant. Numerous males, however, are often produced even in non-apical galls. In such instances conditions in the root may be

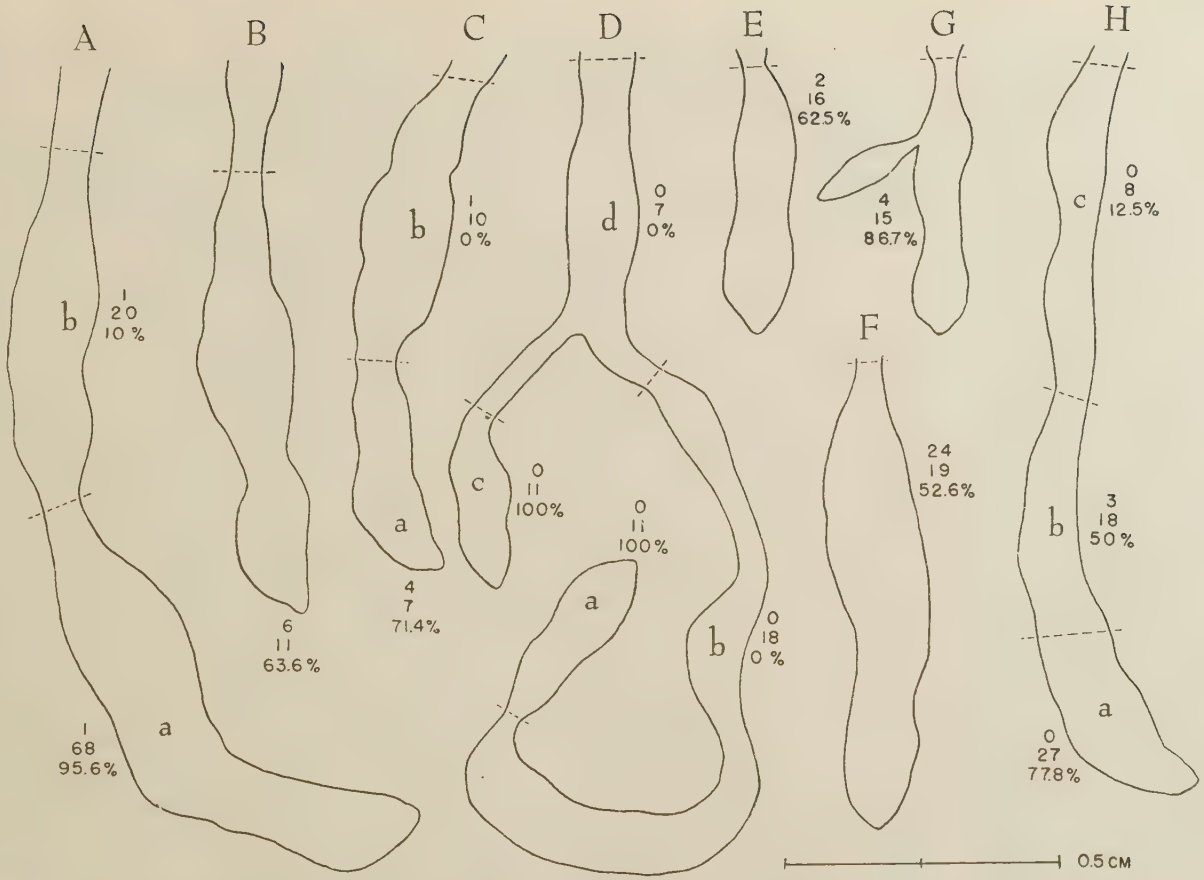

Figure 1. Camera lucida drawings of tomato rootlets infected by *M. incognita*. Upper number = sexually undifferentiated larvae found in each root portion; middle number = sexually differentiated individuals; lower number = per cent of males among the sexually differentiated individuals.

changed by certain factors following infection. Conditions may become unfavorable for development of the nematode when a higher part of the same root is damaged in such a way that it affects food translocation. Unfavorable conditions may also result when larvae of the second generation remain and develop in the same root portion where they were produced.

Male development in incubated roots. In the first generation (Table I, samples 1-5) males obtained from the incubated parts of the roots were more numerous than those found in the stained material. For example, in sample 1, 23 males were obtained from incubated roots, whereas no males were found in the other half of the same sample which had been stained. In sample 2 also the incubated material gave 9 adult males, whereas only one second stage male larva was found in the stained material.

These results were confirmed in additional similar experiments. Under the same environmental conditions and conditions of infection the incubated material always gave numerous males whereas very few or no males were detected in the stained material.

One explanation for this would be that young second stage larvae, still sexually undifferentiated, continued their development in the incubated roots and eventually gave rise to adult males. However, the absence of males in the stained root material of samples 2 to 5, indicates that such development did not occur in plants growing in the greenhouse. The development of males in the incubated roots must have been induced by some factors operative after the samples were taken.

Another explanation would be that some of the individuals listed in Table I as female second stage larvae were in fact male second stage larvae with 2 gonads. This explanation, however, must be rejected because these males should appear as adult males in the stained roots of samples 2 to 5.

A third explanation might be that some of the female second stage lavae in the incubated roots continued developing and through sex reversal completed their development as males. Such males should possess 2 testes since these individuals originally were female larvae with 2 gonads.

To investigate further these possible explanations, another similar experiment was conducted. As host plants in the second experiment, young seedlings of tomato, corn and cotton were used. In addition, old tomato seedlings growing for 5 months in 2″ pots were in-

cluded. Three isolates of *M. incognita* and one isolate of *M. javanica* were used.

The results of the second experiment are shown in Tables II and IV. In samples 1 to 4 (Table II) which were under conditions favorable for development of the nematode (first generation of the parasite on young tomato plants), numerous males were obtained from the incubated material, whereas no males were found in the stained material. This supports similar observations of the first experiment. In addition, most of the males (88-92.5%) possessed 2 testes as it would be expected under the third assumption made previously. These males may have developed from female larvae through sex reversal during the incubation process. In samples 6 and 7 a small percentage (6.7-13.5) of the males obtained from the incubated roots possessed 2 testes. These samples were under conditions unfavorable for development of the nematode, since a second generation of the parasite developed in the same plants. Samples 8 and 9 were also under unfavorable conditions since cotton was only a moderately suitable host for these isolates of the parasite. In these samples the percentage of males with 2 testes was somewhat higher than in samples 6 and 7 but still low in comparison to samples 1 to 4. Finally, sample 10 from old tomato seedlings also gave a low percentage of males with 2 testes.

Hypothesis on mechanism of sex determination, and supporting discussion with respect to experimental data. On the basis of these experimental results the following explanation can be given as to development of males and mechanism of sex determination. Under conditions favorable for the development of the nematode, most of the second stage larvae differentiate as females and develop into adult females with two ovaries. Under unfavorable conditions, most of the second stage larvae differentiate as males and develop into adult males with one testis. When the conditions change from favorable to unfavorable, the second stage female larvae either die or undergo sex reversal and continue their development to adult males which possess two testes.

Under conditions favorable for development of the nematode all or most of the nematode larvae differentiated as females (Tables I and II, samples 1 to 5, stained roots). Males were absent or rare. By decapitating infected plants and incubating the roots, the conditions in the roots (food supply, food composition, aeration, by-products of microorganisms and others) soon changed. Under these conditions most individuals in the incubated roots died. However, many of the

TABLE II

Development of *Meloidogyne incognita* on different hosts at 29° C and numbers of males obtained from infected roots incubated for 40 days in moist chambers at 19° C.

Sample No	Isolate [a]	Host Plant	Development Days [b]	Incubated Roots		Stained roots							
				Males		2nd stage larvae undifferentiated		No females					Males all stages
				Total No	With 2 testes %			Larval stages			Adults		
						Infective	Developed	2nd	3rd	4th	Without eggs	With eggs	
1	1C—1	Tomato	9—10	40	92.5	126	289	61	4	—	—	—	—
2	1C—1	Tomato	13—14	10	90.0	49	12	38	19	9	14	—	2
3	9A—6	Tomato	8—10	92	92.4	159	258	27	—	—	—	—	—
4	9A—6	Tomato	10—12	25	88.0	1	10	68	11	15	—	—	—
5	9A—6	Tomato	22—24	1	100.0	—	5	—	—	—	130	46	—
6	9A—1	Tomato	70	155	13.5	30	45	29	3	15	95	32	135
7	2A—3	Corn	70	95	6.7	22	32	25	1	4	43	14	84
8	1C—1	Cotton	16	27	33.3								
9	9A—6	Cotton	16—18	33	24.2								
10	1C—1	Old Tomato	15	20	15.0								

[a] Single egg-mass isolates from nematode cultures obtained from the following localities: 1C — N. Carolina; 2A — Georgia; and 9A — S. Carolina.

[b] Period from penetration of larvae of the first generation into the roots to incubation or staining.

second stage female larvae underwent sex reversal and were then able to continue their development to adult males. Such males possessed 2 testes and comprised about 90% of the total males (Table II, samples 1 to 4). Second stage larvae which at the time of incubation, or a little later, were sexually undifferentiated continued to develop. Several of them differentiated as males and finally gave rise to very small adult males with one testis. Approximately 10% of the total males were of this type. Others differentiated also as males, but because of insufficient initial development did not reach the adult stage.

Under conditions unfavorable for development of the nematode many of the nematode larvae developed as males. Factors inducing maleness exercised their effect early in the development of the sexually undifferentiated larvae resulting in the differentiation of some of them as males with one testis. They were found in stained roots of samples 8 to 11, Table I and samples 6 and 7, Table II. Similarly, in incubated roots, many adult males or male larvae were present at the time of incubation. These were found later in the incubation period as adult males with one gonad (Table II, samples 6 and 7). In addition several sexually undifferentiated larvae continued their development after incubation. They differentiated as males and gave rise to very small adult males with 1 testis. Males of these 2 categories comprised 86.5-93.3% of the total males obtained from incubated roots. The rest of the males possessed 2 gonads. They derived from second stage female larvae which following incubation underwent sex reversal and then continued their development to adult males with 2 testes.

Anatomical evidence of sex reversal. The hypothesis of sex reversal is supported by the following anatomical studies and observations. Examination of second stage larvae obtained from stained roots of sample 11 of Table I (conditions unfavorable for development of the nematode) revealed peculiarities in gonad development which were not observed in larvae from samples 1 to 3 of Table I, and 1 to 5 of Table II (conditions favorable for development of the nematode). In 9 of the 15 individuals listed as female second stage larvae in sample 11, Table I, the well differentiated female gonad was located far from the posterior end of the body (Fig. 2A), whereas normally it should have moved posteriad earlier in its development (17; Fig. 2A). This retardation of posteriad movement of the gonad is characteristic of male individuals. In some of these individuals it was observed that the gonad consisted of 2 branches as in female larvae, but one of the branches was considerably longer than the other (Fig 2B). These

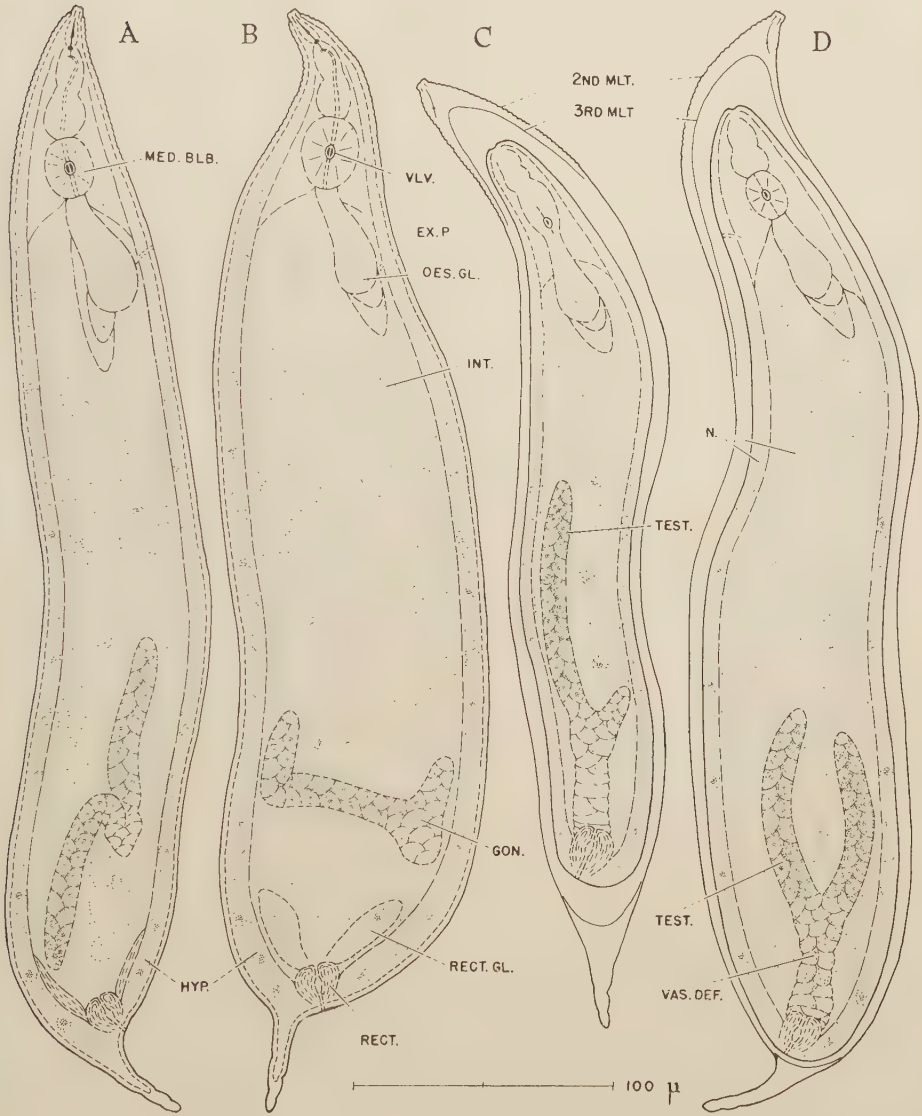

Figure 2. *M. incognita.* A-B — female second stage larvae undergoing sex reversal; C-D — male fourth stage larvae which developed from female larvae through sex reversal (for explanations see text, page 22). Ex. p. = excretory pore; gon. = gonad; hyp. = hypodermis; int. = intestine; med. blb. = median bulb; mlt. = molt; n. = nucleus; oes. gl. = oesophageal gland; rect. = rectum; rect. gl. = rectal gland; test. = testis; vas. def. = vas deferens; vlv. = valve.

cases can be explained on the basis of the hypothesis of sex reversal. These individuals must have been originally differentiated as females with 2 gonads. Changes in conditions in the root may have induced maleness and therefore sex reversal occurred. In the first case, maleness was expressed only as retardation of the posteriad movement of the gonad, whereas in the second case in addition it was expressed as an inhibition of development of 1 gonad branch.

If these abnormalities in gonad development are in fact the result of sex reversal at a critical period during the second stage, one might expect to find similar abnormalities in the incubated roots where sex reversal is artificially induced.

In order to investigate this, the incubated roots of samples 3 to 5 of Table II were examined 20 to 30 days after incubation, i.e. before the males began to leave the roots. All nematodes found in the roots were stained in acid fuchsin lactophenol, mounted on slides and examined under the compound microscope.

The adult males and fourth stage male larvae are shown as males in Table II. In addition to these, however, several second stage abnormal female larvae of the type illustrated in Fig. 2A, B were found as well as some fourth stage male larvae with 2 testes of unequal length (Fig. 2C). These results further support the hypothesis of sex reversal. The abnormal individuals (Fig. 2A - D) can be considered transitional stages in the process of sex reversal from normal female to sex reversed male larvae.

Evidence of male development from sexually undifferentiated larvae in incubated roots. In sample 3 of Table II there were also found in incubated roots 10 very small second stage male larvae possessing one gonad. In the stained material of the same sample no male second stage larvae were present. The supposition, therefore, can be made that these larvae developed during the incubation period from second stage sexually undifferentiated larvae. They differentiated sexually as males, and most of them ceased further development because of insufficient original growth. Others, however, continued their development and were found in the fourth stage or as adult males possessing 1 gonad. They constituted 7.6% of the total males of this sample.

Size of males in relation to their feeding period. The size of adult males was determined by the extent of growth they had reached at the time the food supply was discontinued. This can be demonstrated by data obtained from the first experiment. Thirty males

obtained from incubated roots of samples 7, 8 and 10 of Table I were measured and their average length is given in Table III. It is apparent that average male length increases constantly with length of feeding period of the parasite in roots of the plants. In sample 7 (Table I) all males obtained from incubated roots must have been derived from the development of young sexually undifferentiated second stage larvae or perhaps from a few sexually differentiated se-

TABLE III

Average size of males of *Meloidogyne incognita* obtained from incubated roots. (Sample No corresponds to Table I).

Sample No	Development [a] Days	Probable period of development [b] Days	No males examined	Average length microns	L.S.D. 5%
7	37—42	⟨10	30	769.1	
8	42—47	⟨15	30	1081.3	113.2
10	55—60	⟨28	30	1580.7	

[a] period from penetration of larvae of the first generation into the roots to incubation.

[b] period from penetration of larvae of the second generation into the roots to incubation.

cond stage larvae (see stained material) Their size, therefore, was very small. In sample 10 most of the males derived from either fully developed males existing in the roots at the time of incubation or from fourth or third or well developed second stage male larvae. These individuals had attained their full growth before the roots were incubated. Some males, however, developed from young second stage larvae as in the case of sample 7. These males were small in size and caused a slight decrease in the average length of the males of sample 10.

True males and male intersexes in Meloidogyne javanica. Male intersexes are male individuals that also possess secondary female characteristics. In *M. incognita* males which developed from female larvae through sex reversal can hardly be called intersexes, since they do not possess any character other than the 2 testes corresponding to the 2 ovaries of the female. Male intersexes, however,

exist in *M. javanica*. They are males which in addition to male characters show female characteristics ranging from rudimentary vagina to well developed vulva. According to Chitwood (4), male intersexes in this species constitute the majority of male individuals and true males (non-intersexes) are rare. They all possess 2 testes.

If sex in *M. javanica* is determined by the same mechanism as in *M. incognita*, the following suppositions could be made: (a) under conditions unfavorable for development of the nematode, many true males should develop and should possess only one testis, (b) male intersexes should develop from female larvae through sex reversal and therefore should possess 2 testes.

These assumptions were investigated in the second experiment. The results are presented in Table IV.

TABLE IV

Number of testes in true males and male intersexes of *Meloidogyne javanica* obtained from incubated tomato roots.

Sample No	Development [a] Days	True Males		Male intersexes	
		Total	With 2 testes %	Total	With 2 testes %
1	70	79	19	120	93.4
2	95	34	0	23	78.3

[a] period from penetration of larvae of the first generation into the roots to incubation.

In both samples a considerable number of true males were present in addition to the male intersexes. In sample 2, true males were even more numerous than male intersexes.

The majority of true males possessed one testis whereas most of the male intersexes possessed 2 testes. These results are in agreement with the assumptions. However, true males with 2 testes and intersexes with one testis were also found constituting a small minority. This deviation from the expected can be understood by recognizing that the relative development of several characteristics in nematode intersexes cannot always be correlated. In one case a certain sexual character develops more than another, whereas in a second case the opposite may be true. This is supported by observations in

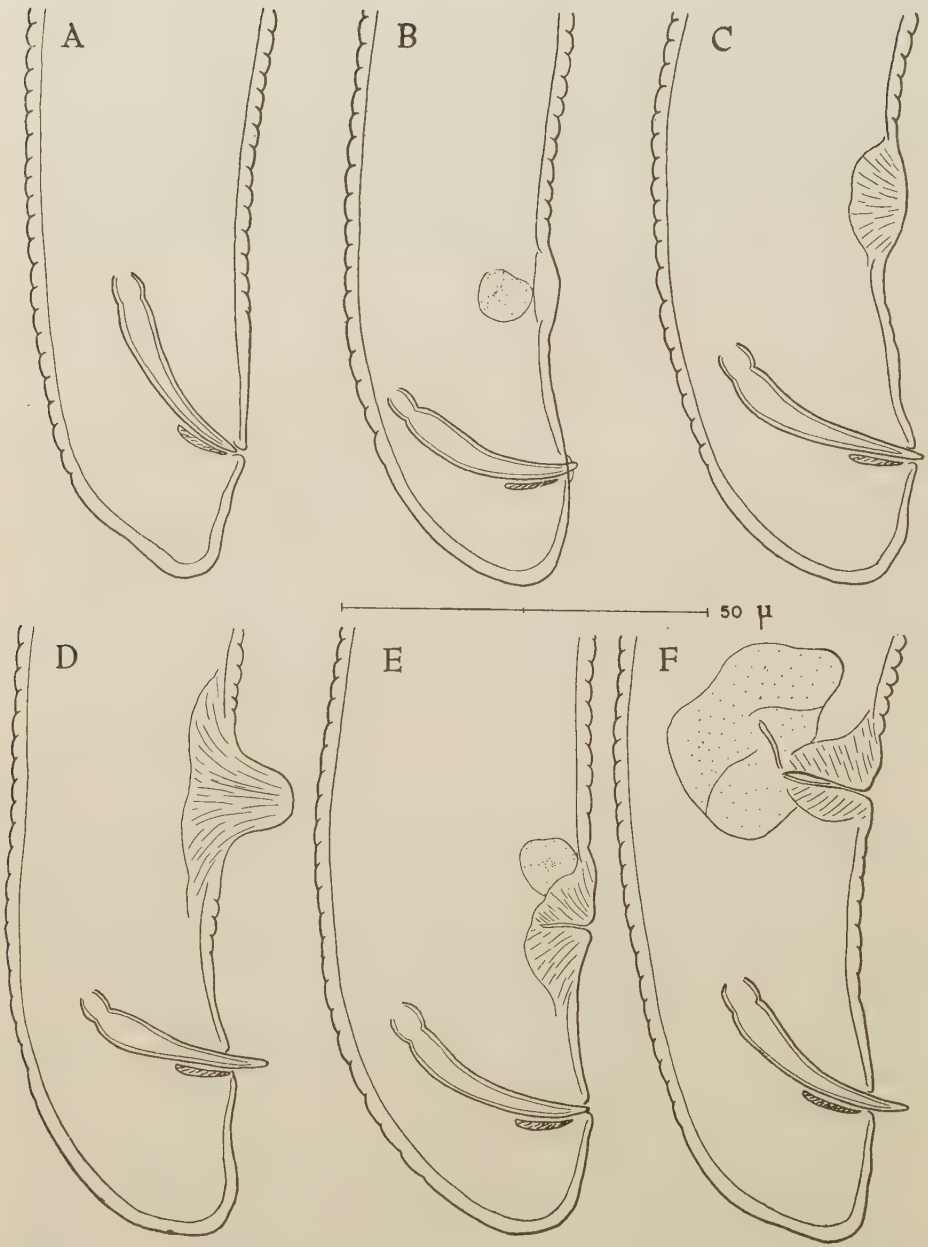

Figure 3. Posterior part of a true male (A) and several male intersexes (B-F) of *M. javanica*.

other species of nematodes producing intersexes (15). It is therefore not necessarily true that individuals with 2 testes must be intersexes or that intersex individuals must always possess 2 testes.

The quantitative type of effect of the factors affecting development of female characteristics in intersex individuals of *M. javanica* is illustrated in Fig. 3. Some intersexual individuals (Fig. 3 B, C) can hardly be distinguished from true males (Fig. 3A). A slight swelling and an interruption of the cuticular annulation in the vulvar region are the only distinguishing characteristics. Individuals with more pronounced intersexuality possess a larger swelling in the vulvar region (Fig. 3D) or a well formed vulva (Fig. 3E) sometimes associated with additional tissues of unknown origin and function (Fig. 3F).

A similar quantitative type of effect can be detected in the development of the testes. Sometimes males and male intersexes may possess 2 fully grown testes or growth of one of them may be inhibited to varying degrees.

It seems, therefore, that both these sexual characteristics are determined on a quantitative basis and development of the one is independent of the development of the other. This agrees with the developmental pattern of intersex characteristics described by Steiner in Mermithidae (15).

DISCUSSION

Males with 2 testes obtained from incubated roots infected by first generation larvae were all small in size. Under natural conditions, however, large males with 2 gonads were also found. In intact plants it is possible that the larvae continue to feed following sex reversal and attain complete growth before they undergo the second molt. Thus they give rise to large males with 2 testes. The third and fourth stage larvae, as has been emphasized by Bird (3), require no food for their development to the adult stage. Although the adult males are mature shortly after the fourth molt, young females require additional feeding before they reach maturity. Females which at the time of incubation are close to maturity can complete their development and start laying eggs. The oviposition perhaps proceeds for several days even under conditions of starvation. In one case it was observed that at the time of incubation only 2% of the adult females in the roots had laid a few eggs each. Fifteen days later 85% of the adult females in the incubated roots possessed well-formed egg masses.

Conditions comparable to root incubation are often present in plants weakened by various types of damage. Under such conditions even susceptible host plants may offer unfavorable environment for the develoment of the parasite. As the environment becomes less favorable the male to female ratio increases. This change in sex ratio will drastically affect the size of the nematode population. Fluctuations of root-knot nematode populations in the field may sometimes be explained on this basis.

There is indication that cotton which is not a suitable host induced maleness. Similar results were obtained with very old plants of a suitable host (tomato, var. Rutgers). Van der Linde (11) observed numerous males on a commercial variety of *Ricinus communis* L. (unsuitable host) and Dropkin (6) found few males on suitable soybean varieties and varying percentages of males on unsuitable varieties. The above together with the effect of crowding of the larvae suggest that maleness may be induced by quantitative and/or qualitative nutritional deficiencies. Further investigations will be necessary before the specific factors influencing the sex of the root-knot nematode larvae are well understood.

Environmental factors may affect sex also in other nematode species which under certain conditions show a high fluctuation in the male to female ratio. This has been proven for some insect parasitic nematodes as *Mermis subnigrescens* (5) and has been suggested for some plant parasitic species like *Heterodera rostochiensis* (7) and *H. schachtii* (2).

The phenomenon is not unique in the nematodes. It has been reported in other animal groups such as the marine worms *Bonellia viridis* Rol. (14) and *Dinophilus* (19), some crustaceans (monstrillids) (13), amphibians (14) and others.

In *Meloidogyne javanica* the mechanism of sex determination and sex reversal seems to be similar to that of *M. incognita*. Some differences, however, must exist with regard to the response of these species to the factors governing sex. Sex reversal, when it occurs, is complete in *M. incognita*. The sex reversed individuals develop as males apparently without any female characters except the 2 gonads corresponding to the 2 ovaries of the female. In *M. javanica*, however, sex reversal may not be complete, thus permitting the limited development of some of the female characters (vagina, vulva) in sex reversed individuals. Such individuals are the so-called male intersexes

which are unique in this species. All the intersexes observed in other nematode species are female intersexes.

In some insect parasitic nematodes of the family Mermithidae intersexuality may be governed by the same mechanism as in *M. javanica*. In both instances nutritional factors influence sex strongly and perhaps exclusively. The total number of the larvae may differentiate as either females or males depending upon the nutritional conditions of the host plant or insect. In both cases also there exists a wide variation in the size of the individuals that is obviously affected by conditions of nutrition. In *Agamermis albicans* males may vary in size from 29 to 80 mm and females from 38 to 320 mm. The nutrition of the host may affect not only the growth of the parasite, but also its physiological balance. Intersexes may develop from female larvae when the nutritional conditions become unfavorable at a stage in differentiation at which complete sex reversal is precluded. The factors for maleness under such conditions may have a slight effect resulting in development of rudimentary male sexual organs on female individuals which thus become female intersexes. Complete or incomplete sex reversal through changes of the environment has been reported in *Xiphophorus helleri* (1), in some species of amphibia (14), and others.

SUMMARY

The mechanism of sex determination and the effect of several factors on the sexual differentiation of larvae of *M. incognita* were studied.

When few nematode larvae entered singly tomato roots, most or all of them developed into females. When large numbers of nematode larvae entered the apical portions of tomato rootlets, growth of the rootlets ceased and apical galls were formed. In the apical galls the nematodes were crowded and under these conditions most or all of them developed into males. It was postulated that conditions unfavorable for the development of the nematode induced maleness.

Galled root portions of tomato plants were removed and divided into 2 representative samples. One sample was stained with acid fuchsin laclophenol, the other was incubated in a moist chamber at 19° C. Numerous males were obtained from the incubated material, whereas, very few or no males were detected in the stained material. In incubated roots all second stage male larvae, as well as many of

the second stage larvae which at the time of incubation were sexually undifferentiated, developed into adult males with one testis. Many of the second stage female larvae, however, underwent sex reversal and then continued their development to adult males with 2 testes. That sex reversal from female larvae with 2 gonads to males with 2 testes actually took place was supported also by anatomical studies.

The average length of males increased constantly with the length of feeding period of the nematodes in the plant roots.

In the case of *M. javanica* true males were found in addition to the male intersexes. True males were abundant under conditions of food stress. Intersexes were apparently derived from second stage female larvae through sex reversal. The majority of true males possessed one testis, whereas most intersexes had 2 testes.

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